SELECTIVE LIGHT ABSORPTION BY THE LENSES OF LOWER VERTEBRATES, AND ITS INFLUENCE ON SPECTRAL SENSITIVITY

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Visual processes in all vertebrates apparently depend upon a group of closely similar carotenoid-proteins. Since the spectral distribution of sensitivity is determined by the absorption spectra of these pigments, it is no accident that most vertebrates are sensitive to approximately the same band of wave-lengths. In man, this range lies between the rough limits of 400 mμ and 700 mμ. As an expression of these limitations, we have come to call wave-lengths longer than 700 mμ "infrared" and those shorter than 400 mμ "ultra-violet."

The long-wave-length limit of sensitivity is relatively inflexible among vertebrates, because the visual pigments so far isolated from retinas do not absorb significantly above 700 mμ. At the other end of the spectrum, however, the limit imposed is of quite a different sort. The visual pigments rhodopsin, porphyropsin and iodopsin (Wald, 1955; Wald, 1939; Wald, Brown and Smith, 1955) all show considerable absorption between 300 and 400 mμ, with a secondary maximum present in this region. In the case of rhodopsin, the absorption at 360 mμ is nearly 30% of that at the 500 mμ maximum; the absorption at 600 mμ, by contrast, is less than 10%. Light of these "ultra-violet" wave-lengths is thus potentially available for utilization in the sensory process.

In the case of human vision, Wald (1952) has shown that the short-wave-length limit at approximately 400 mμ is imposed by selective absorption in ocular tissues. Below 310 mμ, in the region of general protein absorption, almost all light is absorbed by the cornea, since it is the first tissue encountered. The lens, which appears yellow in color (especially in aged persons), is an effective cut-off filter for radiations below 400 mμ. Aphakics (persons with lenses excised for cataracts) tested by Wald could read an optometrist's chart by the isolated 365 mμ line from a mercury arc, under which conditions a normal person could see nothing at all. Although the pigment responsible for the coloration of the human lens—and for its properties as a filter—has not been definitely identified, the indications are that it is a melanin (Gourevitch, 1949).

Walls and Judd (1933) and Walls (1940) attempted a comparative survey of the occurrence of such filtering lenses in other vertebrates. They found yellow lenses in the eyes of some diurnal reptiles (snakes and certain geckoes), lampreys, squirrels, tree shrews and ground squirrels (*Citellus*). None were seen in fishes

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or amphibians; but the yellow perch and the bowfin (*Amia*) both had yellow corneas. Walls believes that the functional advantage in the selective removal of short-wave-length radiations lies in promoting visual acuity. Chromatic aberration, since it increases exponentially as the wave-length decreases, can produce serious errors in the violet and ultra-violet (Wald and Griffin, 1947). It is therefore adaptive for diurnal animals—whose requirement is for acuity and not sensitivity—to remove this region of the spectrum before the light reaches the retina. This idea is supported by Walls' finding that yellow lenses occur only in diurnal forms.

The mere presence of a yellow coloration, however, does not mean that the lens is a successful ultra-violet filter. Many yellow pigments (for example, xanthophyll and carotene) absorb in the blue region of the spectrum and not in the near ultra-violet. Conversely, lack of a visible yellow color does not mean that the lens is not an effective filter for the near ultra-violet. A filter absorbing nothing above 400 mμ but cutting off sharply at 390 mμ, for example, would appear colorless to the human eye, but it would be a powerful aid to visual acuity for the animal possessing it.

The fact that the human eye is a poor instrument with which to assess these properties prompted the present investigations. These experiments are an attempt to measure quantitatively the selective absorption of lenses, comparing those of a wide variety of lower vertebrates. Such measurements may clarify the functional significance of this interesting visual adaptation; in addition, they provide concrete information about an important dimension of sensory capacity in these animals. Some studies on the chemical basis of this selective absorption are also reported. Finally, the influence of these filtering lenses upon spectral sensitivity is assessed by electrophysiological measurements comparing ultra-violet sensitivity in animals with and without their lenses. A preliminary report of some of these experiments has appeared elsewhere (Milkman and Kennedy, 1955).

Methods

For measurements of intact lens absorption, fresh lenses were dissected from the experimental animals and placed in a holder designed to fit a spectrophotometer cuvette. The holder was constructed in such a way that light passed through the lens and out an exit pupil of approximately one mm. diameter, corresponding in position to the central axis of the lens, and thence to the photocell of the spectrophotometer. Thus the absorption of only a small axial section of lens tissue was measured. The lens and holder were immersed in a cuvette filled with mineral oil, which was chosen to match as closely as possible the refractive index of the lens and thereby eliminate errors due to refraction. The mineral oil also prevented the lens from growing opaque during the period of measurement.

A Beckman DU quartz spectrophotometer was used to measure the transmission of the lenses to light of different wave-lengths. Measurements were checked repeatedly, and it was found that the transmission at a particular wave-length did not change significantly during the course of an experiment. A "blank" was used which consisted of an adapter without the lens, suspended in a similar medium. In certain species, the transmission of the cornea was measured using the same apparatus and a similar technique.

In attempts to discover the chemical basis of the selective absorption found in the lenses of certain of the species tested, aqueous extracts were made from large amounts of homogenized lens tissue. Lenses were ground with distilled water in a glass mortar; protein material was removed, either by dialysis, precipitation by heating, or making up the solutions to 50% ethanol.

Extracts were chromatographed on Whatman No. 1 filter paper in butanol-acetic acid-water mixtures (5 : 1 : 4), using ascending strips or cylinders of paper. They were viewed under ultra-violet light from a mercury arc lamp, equipped with a filter which removed almost all visible wave-lengths. The chromatograms were treated in various ways. Some were sprayed with ninhydrin; in others, the spots were cut out and eluted with a small volume of water. All absorption spectra were measured in a Beckman spectrophotometer. In some cases, it was desirable to obtain absorption spectra directly from spots on the paper; these were measured directly in the spectrophotometer, using a "blank" of dry butanol-saturated paper and employing a photo-multiplier attachment for extra sensitivity. Satisfactory spectra could be obtained in this way under conditions when eluted samples might have been too dilute to yield satisfactory measurements.

In order to measure directly the effect of selective lens absorption on spectral sensitivity, the spectral sensitivity function of intact frogs was compared with that of animals deprived of their lenses. Briefly, the technique involved recording the electroretinogram (the slow action potential of the retina) from either the cornea or, in the case of excised eyes, from the vitreous body. Moist cotton wick electrodes were used with a capacity-coupled pre-amplifier and oscilloscope. Monochromatic light produced through interference filters (or, in the case of 365 mμ, by isolation of that mercury arc line) was directed onto the eye of the preparation through a pair of opposed annular wedges which regulated the intensity. The optical system was calibrated with a thermopile and sensitive galvanometer. In each experiment, a certain amplitude of b-wave (the large positive potential of the electroretinogram) was chosen as the "criterion response." The intensity of constant-duration flash necessary to produce a response of this amplitude was then found for each wave-length, and the reciprocals of these "threshold" intensities were plotted as a spectral sensitivity curve. Frogs were curarized and dark-adapted before each experiment. Each wave-length was then tested in turn, with control flashes frequently interspersed to assure that a constant level of sensitivity was maintained. Then the lens was removed and the experiment repeated. In a number of experiments, excised eyes were used instead of intact animals; this procedure proved equally satisfactory.

Results

The lenses of many of the fish and amphibians studied showed marked filtering properties. Representative absorption curves of intact lenses are shown in Figure 2, with Wald's data on the Rhesus monkey lens included for comparison. The species studied are divided into roughly three groups. Members of the first of these possess lenses which, like the human lens, show a cut-off at about 400 mμ, but they are clearly better filters in that their rise in extinction is sharper. As a result of their lack of absorption above 400 mμ, these lenses do not appear yellow. The group includes the yellow perch (*Perca flavescens*), the calico bass (*Pomoxis sparoides*), and the blue-gill sunfish (*Lepomis pallidus*), all common fresh-water

species; the scup (*Stenotomus versicolor*), the summer flounder (*Paralichthys dentatus*), the rudderfish (*Serolia zonata*), and the sea robin (*Prionotus evolans*), all marine species; and the grass frog (*Rana pipiens*). The absorption spectra of lenses in this group are roughly similar (Fig. 1), except for that of the frog, in which the cut-off occurs at a definitely shorter wave-length. This will be discussed below.

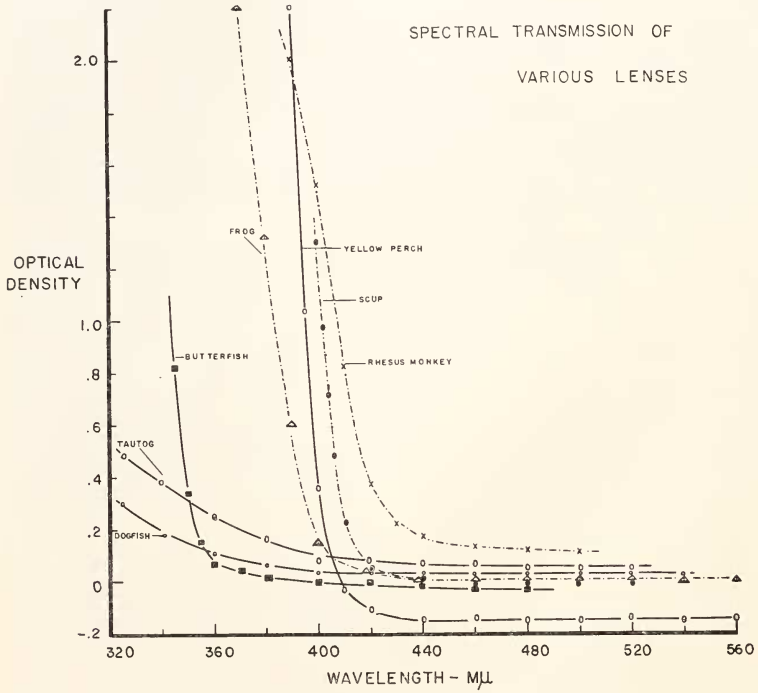

FIGURE 1. Measurements of the spectral transmission of lenses from various lower vertebrates. Data on the lens of the Rhesus monkey from Wald (1949) are included for comparison.

The butterfish (*Poronotus triacanthus*) shows instead a steep rise in lens extinction near 350 mμ, and thus is in a group by itself.

A third group, represented by the tautog (*Tautoga onitis*), the smooth dogfish (*Mustelus canis*) and the toadfish (*Opsanus tau*), all marine bottom species, and the catfish (*Amieurus nebulosus*), a fresh-water bottom scavenger, appear to have only a gradual, slight rise in lens extinction down to 320 mμ. The brook trout (*Salvelinus fontinalis*) is also in this category, but possesses a cornea which shows strong absorption beginning at 400 mμ.

Aqueous extracts of all lenses tested belonging to the first group showed absorption spectra similar to that given in Figure 2, a preparation from lenses of the cod (*Gadus callarias*). Flounder, rudderfish perch, calico bass and sea robin preparations were also tested and found to be spectrally identical; in most further studies, cod lenses were used because they could be obtained in large quantities, fresh, through the courtesy of the Booth Fisheries Corporation of Boston.

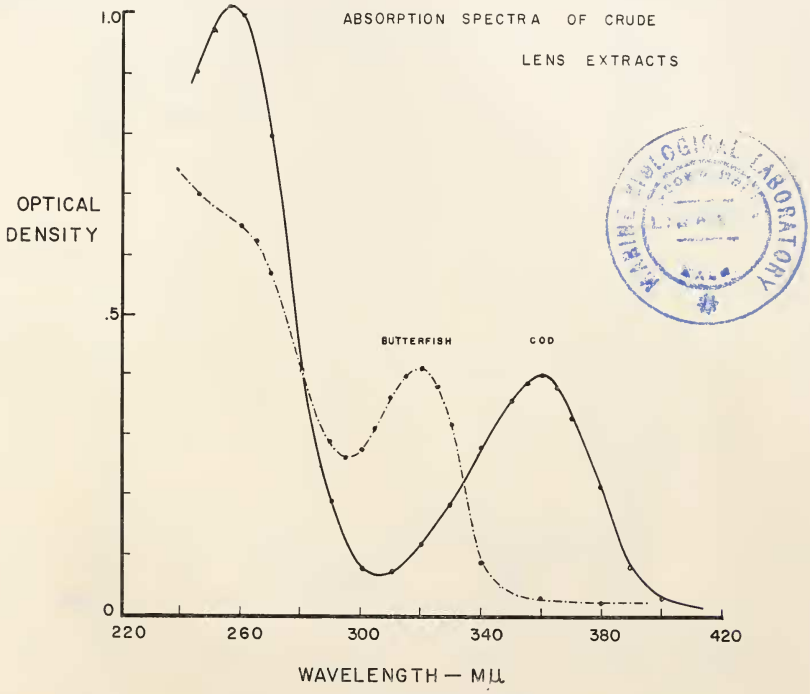

FIGURE 2. Absorption spectra of aqueous extracts from lenses of the butterfish (dotted line) and the cod (solid line). Maxima have been adjusted to same height.

The absorption spectra of these extracts, as shown in Figure 2, show a maximum at 360 mμ, and this absorption band is responsible for the action of the lens as a cut-off filter. In the case of the frog, similar extracts had their maxima at 345 mμ, consistent with the slight displacement of the extinction of intact frog lenses.

Extracts of butterfish lenses (Fig. 2) had absorption maxima at 320 mμ, which explains the fact that intact lenses in this species have their steep rise in extinction at 350 mμ instead of near 400 mμ. No absorption bands between 300 mμ and

400 mμ were found in extracts from lenses which lacked the steep rise in extinction (third group).

The term "pigment" is usually restricted to those substances which absorb in the *human* visible range. This range, however, is restricted by the presence of an ultra-violet-absorbing lens; it is appropriate in this context to refer to the visual

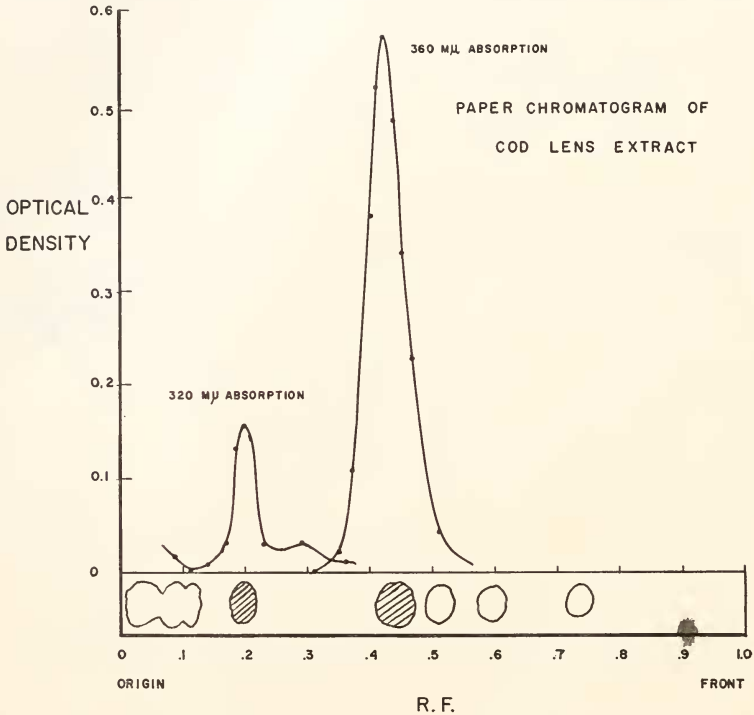

FIGURE 3. Chromatography of cod lens extract. The shaded spots at R. f. 0.19 and 0.43 are, respectively, the presumed oxidation product of 360-pigment and 360-pigment itself: the first spot corresponds with a peak of absorption measured at 320 mμ, and the second with a peak of absorption measured at 360 mμ.

range of vertebrates in general as extending from 310 mμ, below which all ocular tissues absorb strongly, to 700 mμ, the upper limit of visual pigment absorption, provided no special intra-ocular filters intervene. In this sense, then, the filtering substances of the fish lens qualify as pigments, since they absorb light which is visible—though not to humans. We therefore will refer to these substances as pigments, labeling them specifically by their absorption maxima: for example, 360-

pigment for the substance extracted from lenses in the first group, and 320-pigment for the material isolated from butterfish lenses.

After it was found that selective absorption by these lenses had a specific chemical basis, some attempts were made to characterize the substances responsible. Both 360-pigment and 320-pigment are water-soluble, somewhat soluble in methanol and ethanol, and insoluble in all organic solvents tried. They are stable in acid (pH 1), but in alkali (pH 12) they break down slowly and their characteristic absorption bands disappear.

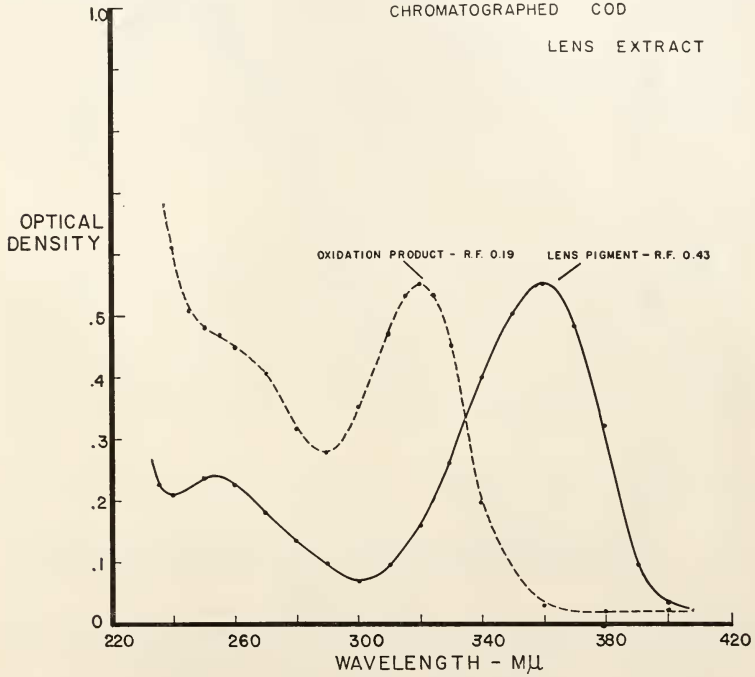

FIGURE 4. Absorption spectra of eluates from the two spots shown in Figure 3. Maxima have been adjusted to the same height.

360-pigment is apparently readily oxidized on standing, or by bubbling oxygen into the solution. The absorption maximum at first shifts from 360 mμ to 320 mμ; this latter band later disappears, and the final product shows only a rising general absorption into the ultra-violet, often developing a tan color suggesting the formation of a melanin-like polymer.

Paper chromatography of lens extracts in butanol-acetic acid-water mixtures (5:1:4) reveals a series of fluorescent and ninhydrin-positive spots. Presumably, a variety of amino acids and polypeptides is present, together with other sub-

stances such as riboflavin. Figure 3 shows the presence of 360-pigment as a ninhydrin-positive, non-fluorescent spot at R. f. 0.43; another spot, yellow-fluorescent and ninhydrin-positive, is present at R. f. 0.19.

Concentrated lens extracts were then chromatographed in streaks, and the bands at R. f. 0.43 were cut out and eluted with distilled water to yield a quantity of purified 360-pigment. When such eluates were allowed to stand overnight at room temperature and rechromatographed, two spots were seen: one was identical

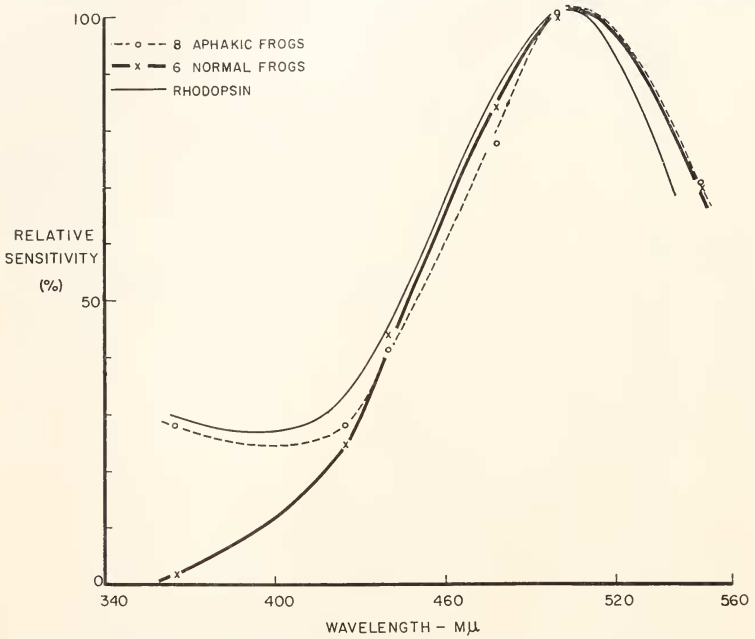

FIGURE 5. Average spectral sensitivity curves at short wave-lengths for normal frogs and frogs with lenses excised, compared with Wald's absorption spectrum for frog rhodopsin.

in position and absorption spectrum to the original eluted spot, and a second was present at R. f. 0.19. This spot was ninhydrin-positive and yellow-fluorescent, and had an absorption maximum at 320 mμ. This is the presumed oxidation product of 360-pigment; as can be seen from Figure 4, it is spectrally similar to 320-pigment from the butterfish.

The ninhydrin-positive nature of both substances in these experiments indicates that an amino group is found in 360-pigment and its derivative. The absorption spectra of chromatographically purified 360-pigment and of its derivative indicate that a second absorption band at 225 mμ is characteristic of both, though in the latter it is present as a shoulder.

When iodine is added to 360-pigment in solution, a quantitative shift of the absorption maximum to 320 mμ is produced. The conversion apparently produces a single product, since there is a clear isosbestic point. It is not certain whether the observed shift is due to saturation of a conjugated double-bond system or another sort of oxidation. The product is spectrally identical with 320-pigment from the butterfish and with the previously-described derivative of 360-pigment; 320-pigment from the butterfish will not react with iodine.

The pigment from the frog lens has an absorption maximum at 345 mμ. It differs from the 360-pigment not only spectrally, but in that it will not add iodine and is acid-unstable and alkali-stable.

The effect of selective lens absorption upon the spectral sensitivity of the frog is shown in Figure 5. The spectral sensitivity function of animals with their lenses removed is in satisfactory agreement with the absorption spectrum of rhodopsin down to 365 mμ in the ultra-violet. Intact frogs, however, begin to show low sensitivity at 425 mμ, and at 365 mμ, sensitivity is only approximately 1% of that at the 500 mμ maximum.

Discussion

It appears from these results that a great many lower vertebrates, as well as mammals, possess an intra-ocular system for filtering out ultra-violet radiations which might otherwise impair visual acuity. A rough correlation is observed here, too, between the existence of such filters and an apparent ecological requirement for acute vision on the part of their possessors. The species found to lack such filters are bottom-feeders like the dogfish which rely primarily on other sensory systems in their feeding. Active, surface-living forms all seem to have filtering lenses; in the butterfish, however, the lens transmits a considerable band of ultra-violet.

The correlation is rather better among higher vertebrates. Squirrels, tree-shrews and primates, among the mammals, are largely diurnal, and have yellow lenses; no nocturnal animal has been found to possess one, and Weale (1953) has shown that the cat lens has a high transmission down to 400 mμ.

In gauging the adaptive value of an intra-ocular filtering mechanism in aquatic animals, a number of complications must be considered. First, though it is generally believed that ultra-violet light penetrates water poorly, the transmission of water for near ultra-violet (350 mμ–400 mμ) is actually quite high compared to light of 550 mμ–600 mμ (Jerlov, 1951). Second, the presence of suspended material increases scattering to a great degree. The consequences of this fact are difficult to ascertain: scattering increases exponentially with decreasing wavelength, so that the presence of suspended matter selectively increases the extinction of short-wave-lengths. However, there are some secondary considerations which cannot be ignored: a plankton-feeding fish, for example, might use ultra-violet sensitivity advantageously to locate concentrated areas of suspended matter (including organisms) by short-wave-length light scattered from them.

Walls (1942) has advanced arguments to support the idea that filtering lenses are a sort of evolutionary "second line" in the battle against chromatic aberration. Retinal cone oil droplets are held to be the usual method of filtering short-wavelength radiations. These are found in turtles and birds (yellow, red, orange and

colorless), amphibians (yellow) and some fishes (colorless). Walls believes that a group which becomes nocturnal in the course of evolution loses its oil droplets; the filtering lens is evolved as a substitute in secondarily diurnal forms. This, he believes, explains the presence of yellow lenses in snakes and diurnal geckoes.

This idea does not seem to explain the situation adequately. Wald and Zussman (1938) have shown that the oil droplets of birds contain three carotenoid pigments, a different one of which is responsible for each color. The yellow one is xanthophyll, which is also responsible for the yellow coloration of the human *macula lutea*. Such droplets are unquestionably filters, but they are not filters designed for removing the ultra-violet. Xanthophyll, for example, has its absorption maximum near 450 mμ, and has declined to a very low absorption in the region of 400 mμ where chromatic aberration begins to become especially serious. The other oil-droplet pigments have their maxima at even longer wave-lengths. In the human eye, in which the cone-rich fovea is equipped with a xanthophyll filter, there is also a yellow lens.

Other evidence, too, contradicts the idea that the filtering lens is functionally identical with the oil droplets and replaces them when the latter are lost in evolution. The frog, which has been shown in these experiments to possess a sharp ultra-violet cut-off in its lens, *also* has yellow cone oil droplets. Finally, we have made observations on the yellow cornea of the yellow perch, and find that it owes its coloration to the presence of (primarily) β-carotene, a carotenoid with nearly the same absorption spectrum as xanthophyll. The perch, too, has a lens filter for ultra-violet. It thus appears that the carotenoid filters in the eyes of vertebrates (oil droplets, yellow corneas, and *macula lutea*) either serve some special function unrelated to the selective absorption of ultra-violet by the lens, or that they are accessory filters which serve to widen the band of short-wave-length absorption. In either case, they are not adaptively equivalent to an ultra-violet filter in the lens.

Evidence suggests that the pigment of the primate lens is a melanin (Gourevitch, 1949). Yellow lenses of other mammals may also owe their coloration to a melanin, although little chemical characterization has been done. The pigment can be extracted by alkali, but not by water (Walls, 1940).

The lenses of the fishes and amphibians studied here owe their selective absorption to an entirely different sort of pigment, which is water-soluble. It has not been possible so far to determine the chemical identity of these lens pigments; their behavior suggests, at least, that 360-pigment from a variety of fish and 320-pigment from the butterfish are closely related chemically.

Not many groups of water-soluble natural compounds show the type of ultra-violet spectrum exhibited by these substances. The two major groups which do are the pteridines (Forrest and Mitchell, 1954a, 1954b, 1955) and some metabolites of tryptophane such as kynurenine. There are chemical similarities between the lens pigments and these two classes of substances, but also some marked differences. At present, there is no definite basis for deciding in which group of compounds the lens pigments belong, although pteridines have been previously isolated from the eyes and integument of fish (Pirie and Simpson, 1946; Hüttel and Sprengling, 1943).

The data on frog spectral sensitivity at short wave-lengths show clearly the large effect which selective lens absorption has on actual visual processes. How-

ever, there is a quantitative discrepancy between *in vitro* measurements of lens absorption and the electrophysiological sensitivity data. Spectrophotometric measurements on the excised frog lens show that it has a transmission at 365 mμ of less than 1% of the incident light. Comparison of the relative sensitivity of normal and aphakic frogs at 365 mμ, however, reveals that the normal animals are about 5% to 10% as sensitive as those lacking lenses. The differences may be explained if it is remembered that in the spectrophotometric measurements, only a small central core of lens tissue was measured; thus, this figure represents the extinction of the longest optical path through the lens. In the intact, dark-adapted animal, with its pupil dilated, light passes through the edges of the lens as well, thus reducing its effectiveness as a filter and accounting for the difference in sensitivity.

The experiments show that the scotopic spectral sensitivity function of frogs clearly agrees with rhodopsin absorption down to 365 mμ, provided no intra-ocular filters intervene, and that an estimate of the effect of such filters in modifying spectral sensitivity may be made by measuring their transmission *in vitro*.

Summary

1. Spectrophotometric studies of fresh intact lenses from a variety of fish and from frogs have shown that they are steep cut-off fibers for ultra-violet radiations, selectively absorbing almost all light of wave-length shorter than 400 mμ.

2. Certain species of fish possess lenses having high transmission in the near ultra-violet, between 320 and 400 mμ; these species must be sensitive to this spectral region, since visual pigments absorb there. Lenses of the butterfish show a steep cut-off at about 350 mμ.

3. There appears to be a correlation between possession of ultra-violet filtering lenses and a requirement for acute vision, supporting the idea that they aid visual acuity by eliminating wave-lengths which produce severe chromatic aberration. Such lenses, however, cannot be regarded as functionally equivalent to such intra-ocular carotenoid filters as retinal oil droplets and *macula lutea* since they absorb in quite different spectral regions. The theory that lens filters are an evolutionary "replacement" for oil droplets in secondarily diurnal animals is thus not in agreement with these findings.

4. Substances responsible for the properties of these lenses as filters have been extracted with water from the lens tissue. Lenses which cut off at 400 mμ yield a substance with an ultra-violet absorption maximum at 360 mμ; those of the butterfish, which cut off at 350 mμ, yield a substance with maximum absorption at 320 mμ. A presumed oxidation product of 360-pigment has been obtained which is spectrally similar to 320-pigment from the butterfish. Both substances have been characterized as to solubility, ultra-violet absorption spectra, and chromatographic behavior, but no definite identification has been made.

5. Comparison of spectral sensitivity in normal frogs and frogs deprived of their lenses has been made by recording the electroretinogram. The results show that the lens has the anticipated effect in restricting short-wave-length sensitivity. In frogs without lenses, scotopic sensitivity is in good agreement with the absorption of rhodopsin down to 360 mμ, while in normal animals sensitivity declines sharply below 400 mμ.

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